REMARKS/ARGUMENTS

Claims 30-79 are active in this application.

Support for the amendment to Claims 39, 40, 41, 42, 64 and 65 is found page pages 26, 28, 31-32 and 33 of the specification.

Claim 79 finds support in original Claim 74.

No new matter is added.

Applicants acknowledge the Examiner's indication that Claims 43-53, 66-73 and 76-78 are withdrawn based on a Restriction requirement and those claims have been duly noted as such. Nonetheless, the non-elected claims (43-58, 66-73 and 76-78) are maintained and not cancelled so that once the elected claims are found to be allowable, the non-elected process claims will be rejoined under the principles set forth in MPEP §821.04: "if applicant elects claims directed to the product, and a product claim is subsequently found allowable, withdrawn process claims which depend from or otherwise include all the limitations of the allowable product claim will be rejoined." Moreover, it is noted that these non-elected claims have been amended in a manner consistent with the elected subject matter so that upon consideration of rejoinder, the claims can be allowed with the elected claims.

As to the restriction of the search of the claims to both of the elected primer pairs (18/19 and 27/28), it is noted that this was an election of species (as opposed to a restriction between groups) with the understanding that once the elected species are to be found allowable, the Office should expand its search to include and allow the non-elected species.

Applicants appreciate the discussion held between the Examiner and their undersigned representative on April 20, 2006. During this discussion, the rejections raised under 35 USC 101 and 112, 2nd paragraph were discussed (see Interview Summary). It is believed that the rejection under 35 USC §101 would be withdrawn in light of the fact that Claims 39 and 65 have been amended to be human cells and which was already apparent

include isolated polynucleotides thereby explicitly involving the "hand of man." Therefore, the claims do not encompass a naturally occurring host cell but rather includes a polynucleotide sequence that had been previously isolated as is specifically recited in the claim. Accordingly, withdrawal of the rejection under 35 USC 101 is requested.

The rejection of Claims 30-42 and 49-65 under 35 USC 112, second paragraph is believed to be no longer applicable in light of the amendments submitted. In particular, while the exact language suggested in the Office Action has not been adopted, the amendments that stated "with the exception that amino acids ___ to ___ are deleted in SEQ ID NO:___" was a subject of discussion during the April 2006 conversation noted above. It is understood that this phrasing is acceptable and addresses this ground of rejection. Accordingly, Applicants request that this rejection be withdrawn.

The rejection of Claims 39-42 and 64-65 under 35 USC 112, first paragraph is believed to be no longer applicable in light of the amendments submitted. In particular, as noted above, these claims have been amended to state that the host cells are "human," which is supported throughout the specification. It is also noted that the interest of using mamamalian cells to express kin17cDNA is described in the attached publication of Kannouche (J Cell Science, 1999, 112, 3215-3224).

Withdrawal of this rejection is requested.

The rejection of Claim 74 under 35 USC 102 (a) or 102(b) in view of Matsuda or EST AI089251 is no longer applicable in light of the amendments submitted. In particular, SEQ ID NO:18 and 19 have been removed from Claim 74. In addition, Claim 79 defines the

polynucleotide as "consisting of" SEQ ID NO:18 or 19 and therefore, the disclosure in these two cited documents is inapplicable to this claim.

Withdrawal of this rejection is requested.

With respect to the Adams AAA reference, the relevant section was separately cited as reference AZ and has been considered.

A Notice of Allowance for all pending claims is earnestly solicited.

Should the Examiner deem that any further action is necessary to place this application in even form for allowance, she is encouraged to contact Applicants' undersigned representative.

Respectfully submitted,

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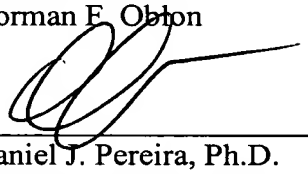
Norman F. Oblon



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Overexpression of kin17 protein disrupts nuclear morphology and inhibits the growth of mammalian cells

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SUMMARY

UVC or ionizing radiation of mammalian cells elicits a complex genetic response that allows recovery and cell survival. *Kin17* gene, which is highly conserved among mammals, is upregulated during this response. *Kin17* gene encodes a 45 kDa protein which binds to DNA and presents a limited similarity with a functional domain of the bacterial RecA protein. Kin17 protein is accumulated in the nucleus of proliferating fibroblasts and forms intranuclear foci. Using expression vectors, we show that overexpression of kin17 protein inhibits cell-cycle progression into S phase. Our results indicate that growth inhibition correlates with disruption of the nuclear morphology which seems to modify the intranuclear network required during the early steps of DNA replication. We report that a mutant encoding a protein deleted from the central domain of kin17 protein enhanced these effects whereas the deletion of the C-terminal domain considerably reduced them. These mutants will be used to elucidate the molecular mechanism by which kin17 protein alters cell growth and DNA replication.

Key words: Replication, Curved DNA, Proliferation, RecA, Intranuclear foci, SV40 T-antigen

INTRODUCTION

The irradiation of mammalian cells provokes a complex response by upregulating several genes, thereby restoring internal homeostasis and allowing cell division. More than 100 genes or proteins participate in this cellular response, the majority of them being involved in general metabolic functions (Friedberg et al., 1995). The role and the respective contribution of most of these genes in cell survival remains to be determined and could be an important clue to understanding vital cellular functions.

We are characterizing *Kin17*, a mouse gene which is upregulated by ionizing or ultraviolet radiation (Biard et al., 1997; Kannouche et al., 1998). *MmKin17* gene encodes a DNA-binding protein, which was initially identified using antibodies raised against the *Escherichia coli* RecA protein. The cross-reactivity is due to a region of 40 residues in the core of kin17 which is homologous to the DNA-binding domain located in the C-terminal end of RecA protein (Aihara et al., 1997; Kannouche et al., 1997; Kurumizaka et al., 1996). kin17 protein possesses three motifs: a zinc finger in the amino-terminal region (aa 27-50), a bipartite nuclear localization signal (aa 239-256), and the core domain homologous to RecA protein (aa 161-201) (Tissier et al., 1995). Although the biological activity of kin17 protein remains to be determined, it has been shown to bind preferentially to curved DNA (Mazin et al., 1994b). Indeed, kin17 protein produced in *E. coli* binds in vitro to the curved DNAs found at the hot spots of illegitimate recombination of human cells (Mazin et al., 1994a). In vivo, kin17 protein produced in bacteria substitutes a bacterial transcriptional regulator called H-NS and mimics its binding to curved DNA. The binding of kin17 protein near promoters induces a topologic change in DNA and leads to gene activation (Timchenko et al., 1996).

In mammalian cells, kin17 protein forms intranuclear discrete foci. This localization reflects a functional compartmentalization of the protein and seems to be relevant to its biological role (Kannouche et al., 1997). Indeed, kin17 protein is accumulated in the nucleus of S-phase cells and after UV or gamma irradiation, suggesting it participates in DNA transactions (Biard et al., 1997; Kannouche et al., 1998). Upregulation of the *Kin17* gene never exceeds 5-fold in cultured cells, and the *Kin17* gene transcript is ubiquitously present at a low level in adult mouse tissues, suggesting that the *Kin17* gene is tightly regulated (Tissier et al., 1996). These observations raise the question of whether overexpression of *Kin17* gene could be deleterious for mammalian cells.

We investigated the effects of kin17 protein on cell growth after transfection of cultured human cells with expression vectors carrying *Kin17* cDNA under the control of a cytomegalovirus (CMV) promoter. We show that the overproduction of kin17 inhibited the proliferation of mouse and human cells. This effect is correlated with changes in nuclear morphology and with a decrease in DNA replication rate. The

deletion of the central domain of kin17 protein enhanced these effects whereas the deletion of the C-terminal domain considerably reduced them. Taken together, our results indicate that overproduction of kin17 protein causes an antiproliferative effect by altering DNA synthesis. These findings suggest that kin17 protein may be involved in DNA replication.

MATERIALS AND METHODS

Cell lines and culture conditions

HeLa (human epithelioid cervical carcinoma) and H1299 (non-small lung cancer) cells grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Mouse BALB/c 3T3 fibroblasts cultured in minimum Eagle's medium (MEM) supplemented with 10% FCS and antibiotics. The H-G1 cell line was derived from HeLa cells by stable transfection of p205-GTI vector (Stary and Sarasin, 1992). A single copy of the plasmid p205-GTI is integrated in H-G1 cells in the chromosome no. 8. H-G1 cells were routinely cultured in DMEM supplemented with 10% FCS and antibiotics, in the presence of 500 μg/ml G418 (geneticin, Gibco, BRL). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Expression vectors

cDNAs corresponding to the open reading frame (ORF) of *Kin17* gene (*Kin17*) or to a deletion from nucleotides 412 to 705 (*Kin17ΔHR*) or from nucleotides 854 to 1034 (*Kin17ΔCT*) were placed under the control of the cytomegalovirus (CMV) immediate early promoter carried by the vector pCMVDT21 (Bourdon et al., 1997). The properties of the encoded proteins have been previously described and are summarized in Table 1 (Kannouche et al., 1997). The plasmid pKMT11 carries a defective origin of replication of simian virus 40 (SV40) and contains the gene encoding large T-antigen under the control of metal ion-inducible mouse metallothionein promoter (Gerard et al., 1985; Stary et al., 1989).

Colony formation assay

BALB/c 3T3 cells were co-transfected with 0.4 μg of pEGFP-N1 DNA, a plasmid coding for the enhanced green fluorescent protein (EGFP) and carrying the neomycin-resistant gene (Clontech) and 4.0 μg of one of the following vectors encoding: (i) kin17 protein (pCMVKin17), (ii) kin17ΔHR protein (pCMVKin17ΔHR), (iii) kin17ΔCT protein (pCMVKin17ΔCT), or (iv) a plasmid lacking the abovementioned ORFs (pCMVDT21). Transfection was carried out with lipofectamine (Life Technologies). For all the plasmids tested, we routinely observed at least 10% transfection. Under our co-transfection conditions, around 80% of the 10% of transfected cell produced both EGFP and kin17 proteins. Transfection efficiency was checked using fluorescence microscopy 20 hours later to detect the EGFP protein. Forty hours after transfection, cells were plated in triplicate at 10^5 cells per 60 mm plate in selected medium containing G418 antibiotic at a final concentration of 800 μg/ml. Drug selection was continued for 2 weeks. The plates were then stained with methylene blue and the colonies containing more than 50 cells were counted. The same experimental procedures were followed using HeLa cells and H1299 cells except that the final concentration of G418 in selected medium was 600 μg/ml for H1299 cells.

Immunofluorescence microscopy

For the visualization of kin17 and large T-antigen proteins, transfected cells were fixed with methanol/acetone (30%/70%, v/v) for 10 minutes at −20°C and rehydrated in phosphate buffered saline (PBS) for 15 minutes at room temperature as previously described (Kannouche et al., 1997). For the detection of other proteins, cells were first permeabilized with 0.5% Triton X-100 for 5 minutes on ice and fixed for 30 minutes with 3% paraformaldehyde in PBS. Cells were then incubated for 60 minutes at room temperature with the primary antibodies diluted as indicated in PBS containing 3% bovine serum albumin (BSA). After extensive washing in PBS, cells were incubated for 45 minutes at room temperature with secondary antibodies. These included Cyanine 2 ($Cy^{TM}2$)-conjugated affiniPure goat anti-rabbit IgG or $Cy^{TM}2$-conjugated affiniPure goat anti-mouse IgG diluted 1:500 in PBS + 3% BSA (Jackson ImmunoResearch Laboratories, Inc), Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG diluted 1:500 in PBS + 3% BSA (Sigma). After washing three times for 5 minutes with PBS, cells were counterstained with 0.2 μg/ml 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) and mounted with Glycergel (Dako). Under our experimental conditions, no significant signal attributable to secondary antibody alone was detected.

Double immunostaining for detection of 5-bromo-2′-deoxyuridine (BrdU) and kin17 protein was taken from the method of Leonhardt et al. (1992). Simultaneous visualization of kin17 protein and sites of new DNA synthesis involved growth of cells in 10 μg/BrdU for 1 or 16 hours. Fixation and staining for kin17 were performed as described above. After the final wash, antibodies were fixed with 2% formaldehyde in PBS for 10 minutes. The coverslips treated with 2 N hydrochloric acid (HCl) at 37°C for 20 minutes were neutralized in 0.1 M borate buffer, pH 8.5. After washing in PBS, cells were incubated for 60 minutes at room temperature with the fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (Boehringer Mannheim). Cells were observed and photographed with a Zeiss Axiophot2 Photomicroscope carrying the ATO-ARC device which allowed observation of the preparations using a low UV intensity. Under these conditions, we visualized only transfected cells expressing relatively high levels of kin17 protein, as previously described (Kannouche et al., 1997).

Antibodies

kin17 protein was detected by indirect immunofluorescence using a rabbit polyclonal antibody directed against bacterially expressed mouse kin17 protein (pAb2064, 1:200) or rabbit polyclonal antibody directed against bacterial RecA (pAbanti-recA, 1:300). The properties and specificity of these antibodies have been previously described (Kannouche et al., 1997). BRCA1 protein was visualized using mAb SD118 (1:50, generously provided by Dr J. Feunteun), Rad51 protein was detected using the affinity-purified rabbit polyclonal antibody (1:50, kindly provided by C. Radding) (Haaf et al., 1995), DNA-polymerase α protein was visualized using mAb 4E9 (1:2, a gift from Dr E. Weiss) and large T-antigen was revealed using mAb 416 1:10 (Yewdell et al., 1986).

Table 1. Description of the truncated forms of mouse kin17 protein

Protein name	Deleted residues	Lacking domain	Nuclear distribution	
			Foci	Large structures
kin17	–	–	++	++
kin17ΔZF	From aa 6 to 70	Zinc finger	++	+
kin17ΔHR	From aa 129 to 228	RecA homologous region	–	+++
kin17ΔCT	From aa 281 to 391	C-terminal end	+	–

Induction of large T-antigen-dependent DNA replication

H-G1 cells were transfected with the pKMT11 plasmid DNA carrying the ORF coding for the large T-antigen. The produced large T-antigen initiated the SV40 DNA replication. The extrachromosomal DNA of low molecular mass was recovered sixty hours after transfection and analyzed as described below.

Plasmid recovery and DNA analysis

The extrachromosomal DNA of low molecular mass was purified from transfected H-G1 cells by small-scale alkaline lysis derived from the method previously described (Stary and Sarasin, 1992). Briefly, 10^6 cells were pelleted and resuspended in 0.1 ml of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, followed by addition of 0.2 ml of 0.2 M NaOH, 1% SDS. After 5 minutes at room temperature and addition of 0.1 volumes of 3 M sodium acetate, the DNA was precipitated with 0.7 volumes of isopropanol. The DNA pellet was rinsed with 70% ethanol, dried and dissolved in 50 µl H_2O. After RNAse A digestion for 30 minutes at 37°C, DNA extracts were digested with *Pvu*II restriction enzyme and analyzed by Southern blotting using Hybon™-N+ as membrane (Amersham, Life Science). The filters were hybridized with the radiolabeled *Pvu*II fragment (300 bp) of p205-GTI plasmid as previously described (Stary et al., 1989). The autoradiographic signals were scanned in an AGFA ARCUS TM plus scanner (AGFA-GEVAERT).

RESULTS

Overexpression of kin17 protein inhibits cell growth of cultured mammalian cells

The *MmKin17* gene is upregulated during cell proliferation: its transcripts increase to maximum levels during the G_1/S transition of the cell cycle (Kannouche et al., 1998). We sought to determine whether overexpression of *MmKin17* gene influences cell growth. In a first set of experiments, we co-transfected the pCMVDT21-derived expression vectors carrying the *MmKin17* cDNA together with the plasmid pEGFP-N1 coding for the enhanced green fluorescent protein (EGFP) and G418-resistance genes into BALB/c 3T3, HeLa or H1299 cells. In parallel, we co-transfected mutants of *Kin17* cDNA coding for truncated proteins lacking functional domains: a core region homologous to RecA protein (kin17ΔHR) or the C-terminal domain (kin17ΔCT).

In order to test the effect of kin17 protein overexpression on cell growth, we used the stable colony formation assay. We co-transfected mouse BALB/c 3T3 fibroblasts with the CMV-based plasmids and pEGFP-N1, selected G418-resistant clones over two weeks and scored only clones formed by more than 50 cells. We carried out a co-transfection with pEGFP-N1 in order to follow the formation of positive clones using an inverted epifluorescent microscope. We observed that cells transfected with the wild-type *MmKin17* or *MmKin17ΔHR* cDNA formed 10- and 5-fold fewer colonies, respectively, than the parental vector missing the *MmKin17* ORF (Fig. 1A and B). In contrast, the clones scored after transfection of the *MmKin17ΔCT* cDNA were comparable in number to those produced by the vector alone indicating that the overproduction of kin17ΔCT protein did not affect the clonogenic growth (Fig. 1A and B). We conclude that in mouse cells, the full-length *MmKin17* or the *MmKin17ΔHR* cDNA behaves similarly, decreasing the colony formation capacity of the transfected cells.

Considering that the human and the mouse kin17 proteins are 92% identical (P. Kannouche et al., unpublished results), we tested whether the *MmKin17* cDNA and its derived mutants would produce the same effect when transfected in human cells. Introduction of these CMV-based plasmids in HeLa and H1299 cells also decreased the number of drug-resistant stable clones (Fig. 1A and B). Thus, we failed to establish BALB/c 3T3, HeLa or H1299 cells producing kin17 protein in more than 5 independent experiments. Furthermore, it has been reported that EBV expression vectors containing the *MmKin17* cDNA and transfected in HeLa or H1299 cells produced a very similar decrease in colony formation, thereby ruling out the possibility that the decrease is due to the plasmids used as expression vector and indicating that the decrease is strictly dependent on the presence of the ORF coding for kin17 protein (Biard et al., 1999) We conclude that (i) the overexpression of kin17 protein in mammalian cells decreases clonogenic growth, and (ii) the essential element responsible for this decrease may be localized in the C-terminal end of kin17 protein.

Ectopic expression of kin17 protein deforms the nuclear morphology of HeLa cells

To characterize further the basis of the anti-proliferative effect produced by high amounts of kin17 protein, we followed the morphology of HeLa cells by indirect immunofluorescence and by phase contrast microscopy between 20 and 30 hours after a transient overexpression of the mouse kin17 protein. We observed two major distribution patterns of kin17 protein depending on the amount of protein produced. 'Pattern A' corresponded to a distribution in discrete foci of about 0.1-0.5 µm diameter throughout the nucleoplasm, similar to the localization of endogenous protein in human cells (Fig. 2A; P. Kannouche et al., unpublished results). Phase contrast microscopy showed that cells displaying pattern A had a morphology very similar to that of non-transfected cells (Fig. 2B). 'Pattern B' was characterized by the formation of large intranuclear structures of 5-10 µm that seem to correspond to protein aggregates (Fig. 2C). Cells presenting pattern B displayed deformations of nuclear morphology (called here DNM) as judged by phase contrast microscopy (Fig. 2D). We observed the DNM of transfected cells before the fixation and indirect immunofluorescence, thus confirming that these nuclear alterations were produced by the overexpression of kin17 protein and not by a structural modification due to the immunostaining procedures. The nuclei of cells displaying DNM failed to present any feature similar to apoptosis after DNA staining with DAPI (data not shown). These results indicate that the overexpression of kin17 protein can alter the nuclear morphology of HeLa cells.

By contrast, cells expressing kin17ΔCT protein displayed a staining that was distributed through the nucleoplasm with the presence of few discrete foci (Fig. 2G). These cells had a normal nuclear morphology as judged by phase contrast microscopy and did not present any detectable DNM (Fig. 2H).

We conclude that (i) the DNM are related to the localization of kin17 protein in large intranuclear structures, (ii) the C-terminal region of kin17 protein is essential for the formation of these aggregate-like structures, and (iii) the presence of DNM seems to correlate with the inhibition of cell growth described in Fig. 1.

*Mm*Kin17ΔHR cDNA behaves like a dominant mutant

The kin17ΔHR protein never formed the discrete foci observed for kin17, kin17ΔZF and kin17ΔCT proteins (Kannouche et al., 1997). Indeed, kin17ΔHR protein always formed the pattern B distribution (Fig. 2E). The cells producing this truncated protein systematically presented DNM as detected by phase contrast (Fig. 2F).

Considering that kin17ΔHR protein lacks a 99-amino-acid region in the core of kin17 protein, we assumed that this region is involved in intermolecular interactions which mediate the formation of the small foci. It could be that kin17ΔHR protein did not properly interact with the normal network of nuclear proteins. Indeed, we have previously shown that kin17ΔHR protein binds tightly to nuclear components of high molecular mass (Kannouche et al., 1997). Our results show that (i) the core of kin17 protein may play a crucial role in the ability to form small foci, (ii) the deletion of the RecA homologous region enhances the formation of DNM. These observations reinforced the idea that the presence of DNM is related to the inhibition of cell growth, as mentioned above (Fig. 1).

Overexpression of kin17 protein inhibits S-phase progression in HeLa cells

The expression of *Mm*Kin17 gene is maximal at the G_1/S boundary (Kannouche et al., 1998). Considering that the exogenous kin17 protein reduced clonogenic survival, we hypothesized that the overexpression of kin17 protein affects S-phase progression in cells and DNA replication. We tested this idea by monitoring BrdU incorporation in cells expressing kin17 protein or its truncated forms by double indirect immunofluorescence assay.

Thirty-two hours after transfection of *Mm*Kin17 cDNAs, cells were pulse-labeled for 1 hour with BrdU. Alternatively they were incubated for 16 hours with BrdU to allow the majority of the cells to undergo a complete cell cycle and to visualize the distribution pattern of the newly synthesized DNA. We then used indirect immunofluorescence to count the BrdU (+) and BrdU (-) cells among the cells expressing the exogenous protein. Overproduction of kin17 or kin17ΔHR proteins prevented incorporation of BrdU (Fig. 3A.a-c and A.e-g and Table 2). 80% of cells producing kin17 in pattern B distribution were BrdU (-). 80% of cells expressing kin17ΔHR protein were

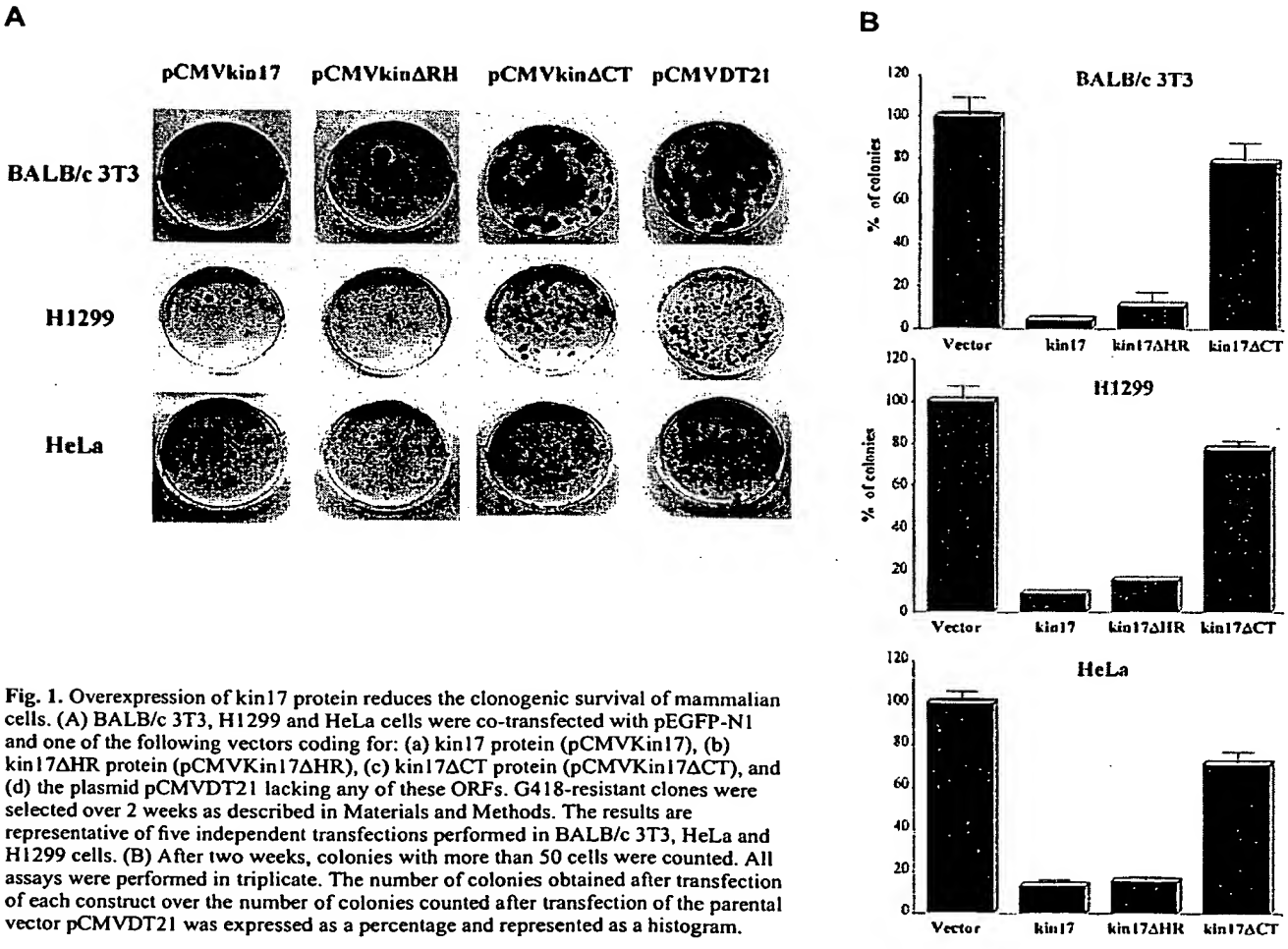

Fig. 1. Overexpression of kin17 protein reduces the clonogenic survival of mammalian cells. (A) BALB/c 3T3, H1299 and HeLa cells were co-transfected with pEGFP-N1 and one of the following vectors coding for: (a) kin17 protein (pCMVKin17), (b) kin17ΔHR protein (pCMVKin17ΔHR), (c) kin17ΔCT protein (pCMVKin17ΔCT), and (d) the plasmid pCMVDT21 lacking any of these ORFs. G418-resistant clones were selected over 2 weeks as described in Materials and Methods. The results are representative of five independent transfections performed in BALB/c 3T3, HeLa and H1299 cells. (B) After two weeks, colonies with more than 50 cells were counted. All assays were performed in triplicate. The number of colonies obtained after transfection of each construct over the number of colonies counted after transfection of the parental vector pCMVDT21 was expressed as a percentage and represented as a histogram.

also BrdU (-) (Table 2). As expected, all these cells presented DNM (Fig. 3A.d and A.h).

Conversely, cells expressing kin17ΔCT protein or displaying the pattern A distribution for kin17 protein underwent DNA replication as visualized by anti-BrdU staining at rates similar to those observed in nontransfected cells (Fig. 3A.a-c and A.i-k and Table 2). Considering that kin17ΔCT protein always displayed a homogenous nuclear distribution and that these cells never presented DNM, we conclude that the reduction in DNA synthesis rate is correlated with the incapacity to form the normal distribution.

The fact that cells overexpressing kin17 or kin17ΔHR proteins did not incorporate BrdU suggested that these cells were blocked before the entry in S phase or in G_2 phase. To verify whether cells displaying DNM were arrested before the S phase, we examined the distribution of BRCA1, rad51 and DNA-polymerase α, three proteins known to have a well-characterized intranuclear dot-like distribution pattern in nuclei of S-phase cells (Scully et al., 1997; Tashiro et al., 1996; Nakamura et al., 1984). Using indirect immunofluorescence, we analyzed HeLa cells 24 hours after transfection with pCMVKin17. More than 90% of cells displaying DNM did not present any staining for BRCA1, rad51 or DNA-polymerase α, indicating that these cells were not in the S phase (Fig. 3B). Moreover, since the normal cycling-cell, BRCA1 and DNA-polymerase α proteins are detectable in G_2 phase (Scully et al., 1997; Nakamura et al., 1984), the absence of staining ruled out the possibility of a G_2 arrest. These results further confirm that cells displaying DMN do not undergo DNA synthesis and support the idea that the inhibition takes place before the entry into S phase. Taken together, our results indicate a correlation between the intranuclear distribution of kin17 protein, the formation of the DNM, the decrease in DNA replication and the inhibition of cell proliferation.

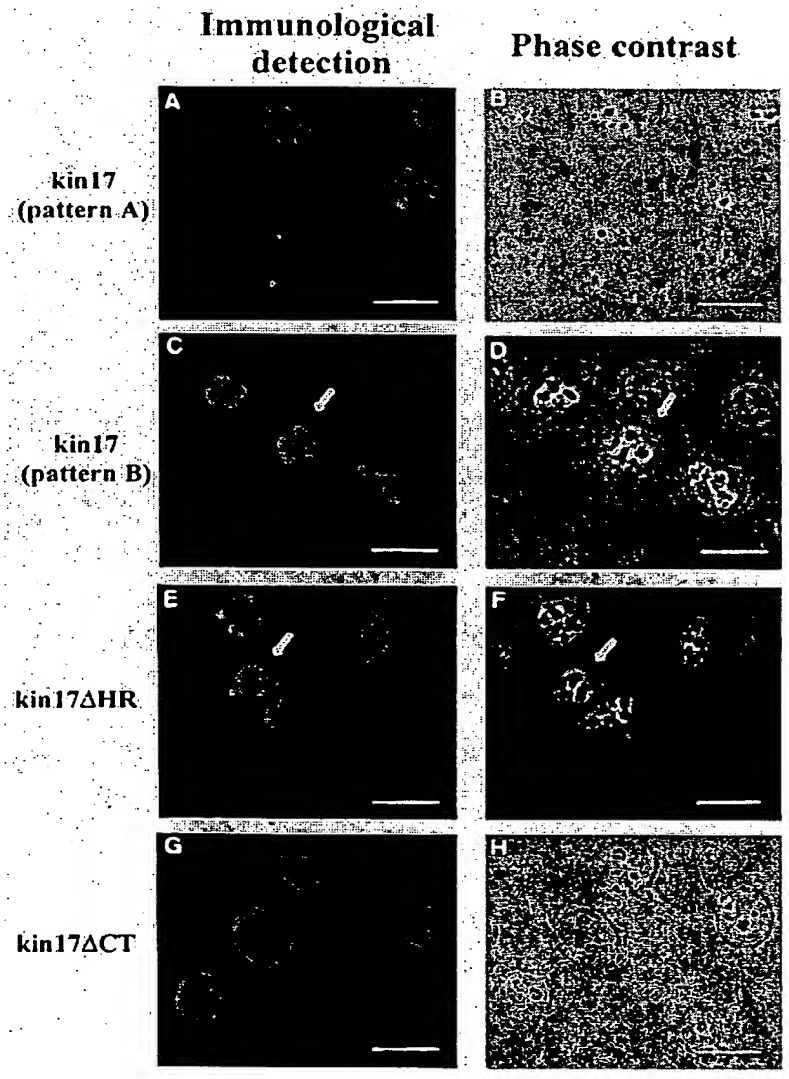

Fig. 2. Cells expressing kin17 protein at a high level or kin17ΔHR at any level exhibit deformation of nuclear morphology (DNM). HeLa cells transfected with pCMVKin17, pCMVKin17ΔHR or pCMVKin17ΔCT were fixed twenty four hours later. Cells were then permeabilized and immunostained with anti-kin17 antibody (pAb2064, A,C,E) or with anti-RecA antibody (pAbAnti-RecAG) as described in Materials and Methods. The same field is shown by phase contrast (B,D,F,H). Arrows point to the deformation of nuclear morphology (DNM) in cells displaying the pattern B distribution for kin17 or kin17ΔHR proteins (C,D,E,F). Bar, 20 μm.

Table 2. Overexpression of kin17 protein in HeLa cells inhibits cellular DNA replication

		% of BrdU (+) cells transfected with			
		pCMVKin17‡			
	Non transfected cells*	Pattern A	Pattern B	pCMVKinΔHR‡	pCMVKinΔCT‡
BrdU incubation of 1 hour					
Exp 1	35	44	0	0	33
Exp 2	25	23	0	0	20
Exp 3	30	30	0	0	28
BrdU incubation of 16 hours					
Exp 1	95	60	19	n.d.	52
Exp 2	75	n.d.	n.d.	17	46
Exp 3	80	59	18	21.5	50
Exp 4	70	53	12	9	47

HeLa cells transfected with the indicated plasmids were cultured in the presence of BrdU for 1 or 16 hours and then immunostained to visualize exogenous kin17 protein (or mutated forms) and BrdU incorporation as described in the text and in the legend of Fig. 3.

*Cells BrdU (+) were scored among 300 cells that did not express exogenous protein, and the number was expressed as a percentage.

‡Cells BrdU (+) were scored among the 200 cells overexpressing kin17 protein (or a derived mutant) and the number was expressed as a percentage.

n.d.: not done.

Expression of kin17ΔHR protein alters chromatin organization

It has been demonstrated in vitro that kin17 protein preferentially binds to curved DNA usually found in illegitimate recombination junctions or at the origins of DNA replication (Deb et al., 1986; Hagerman, 1990; Mazin et al., 1994b). In vivo, the overproduction of kin17 or kin17ΔHR proteins alters the nuclear morphology. We assume that these nuclear deformations are associated with a modification of chromatin organization. To test this hypothesis, we analyzed by double-indirect immunofluorescence the fraction of less than 20% of cells that expressed kin17ΔHR protein and incorporated BrdU. In these latter cells, we observed that the green fluorescence due to BrdU incorporation was concentrated in particular regions of the nucleoplasm, associated with the large structure formed by kin17ΔHR protein (Fig. 4A and B). This pattern was clearly different from the uniform distribution characteristic of nontransfected cells (Fig. 4B). The signals corresponding to BrdU and kin17ΔHR co-localized as judged by the superposition of the red kin17 staining and green BrdU fluorescence (data not shown), suggesting that in vivo kin17ΔHR protein alters the chromatin organization.

Kin17 protein reduces the formation of extrachromosomal DNA generated by T antigen-dependent DNA replication

Since more than 80% of cells displaying DNM were BrdU (-), we investigated whether overexpression of kin17 protein affects the DNA synthesis that is independent on the cell cycle. We therefore tested if in vivo kin17 protein reduced SV40 DNA replication initiated by the large T-antigen. We used H-G1 cells that have a unique copy of the p205-GTI vector which carries an active SV40 DNA replication origin integrated into their genome (Stary et al., 1989; Stary and Sarasin, 1992). The introduction of the non-replicating vector pKMT11 in H-G1 cells transiently increased the large T-antigen concentration initiating DNA replication from the SV40 origin and leading to the formation of low M_r episomal DNA molecules (Stary and Sarasin, 1992). We co-transfected pKMT11 plasmid and the vector expressing (or not) kin17 protein (pCMVKin17 and pCMVDT21, respectively). We recovered extrachromosomal DNA sixty hours after transfection and analyzed it by Southern blot using a DNA probe recognizing the episomal DNA. As expected, the production of large T-antigen led to the formation of low M_r episomal DNA 60 hours after transfection with pKMT11 plasmid alone (Fig. 5A, lane e). The presence of kin17 protein decreased the signal corresponding to episomal DNA (Fig. 5A, lane c). In contrast, when cells were co-transfected with pCMVDT21, the signal intensity was clearly detectable (Fig. 5A, lane d), suggesting that the presence of kin17 protein prevents SV40 origin-dependent replication. Alternatively, the decreased amounts of extra chromosomal DNA might be due to the fact that kin17 protein repressed the large T-antigen synthesis. We checked this possibility by verifying that co-transfected cells expressed both kin17 and large T-antigen proteins. We found by double-indirect immunofluorescence that more than 60% of transfected cells expressed the two proteins. The staining of large T-antigen was uniformly distributed but also associated with kin17 foci, more visible when we merged the red kin17 signal and the large T-signal (Fig. 5B). These results rule out the possibility that kin17 protein alters the SV40 large T-antigen expression and show that the two proteins co-localize in some regions of the nucleus. Taken together, our observations suggest that high levels of kin17 protein reduce SV40 origin-dependent DNA replication.

DISCUSSION

We show that sustained overexpression of kin17 protein results in inhibition of clonogenic survival. This anti-proliferative effect of the exogenous kin17 protein is correlated with alterations in the nuclear morphology of HeLa cells and with reduced DNA replication rates.

The overexpression of proteins may reveal important biological effects that could point to their physiological role. Indeed, overproduction of mammalian nuclear proteins that inhibit cell proliferation, like p53, BRCA1, and AP2, has been widely used to characterize their biological function (Braithwaite et al., 1987; Somasundaram et al., 1997; Zeng et

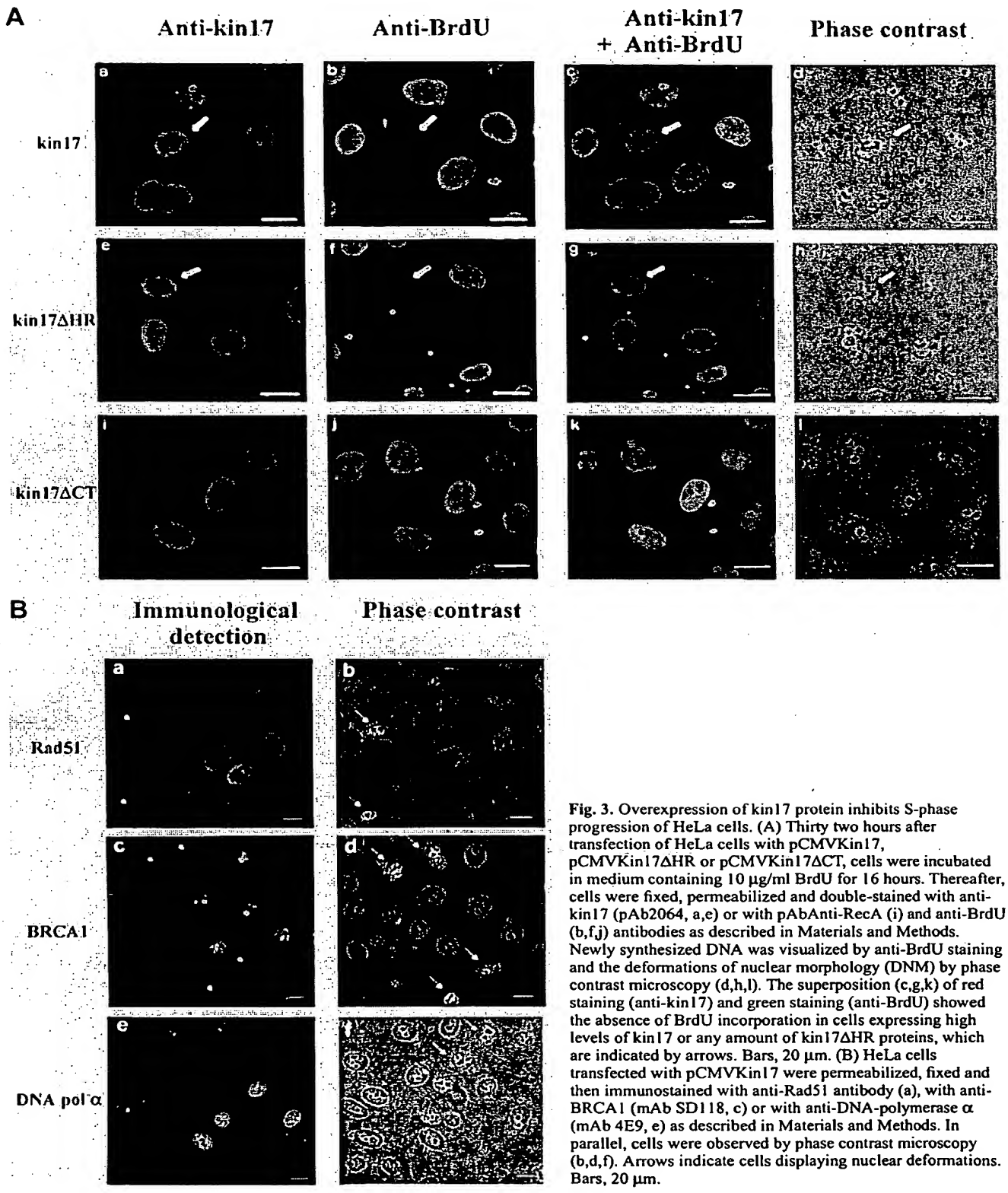

Fig. 3. Overexpression of kin17 protein inhibits S-phase progression of HeLa cells. (A) Thirty two hours after transfection of HeLa cells with pCMVKin17, pCMVKin17ΔHR or pCMVKin17ΔCT, cells were incubated in medium containing 10 μg/ml BrdU for 16 hours. Thereafter, cells were fixed, permeabilized and double-stained with anti-kin17 (pAb2064, a,e) or with pAbAnti-RecA (i) and anti-BrdU (b,f,j) antibodies as described in Materials and Methods. Newly synthesized DNA was visualized by anti-BrdU staining and the deformations of nuclear morphology (DNM) by phase contrast microscopy (d,h,l). The superposition (c,g,k) of red staining (anti-kin17) and green staining (anti-BrdU) showed the absence of BrdU incorporation in cells expressing high levels of kin17 or any amount of kin17ΔHR proteins, which are indicated by arrows. Bars, 20 μm. (B) HeLa cells transfected with pCMVKin17 were permeabilized, fixed and then immunostained with anti-Rad51 antibody (a), with anti-BRCA1 (mAb SD118, c) or with anti-DNA-polymerase α (mAb 4E9, e) as described in Materials and Methods. In parallel, cells were observed by phase contrast microscopy (b,d,f). Arrows indicate cells displaying nuclear deformations. Bars, 20 μm.

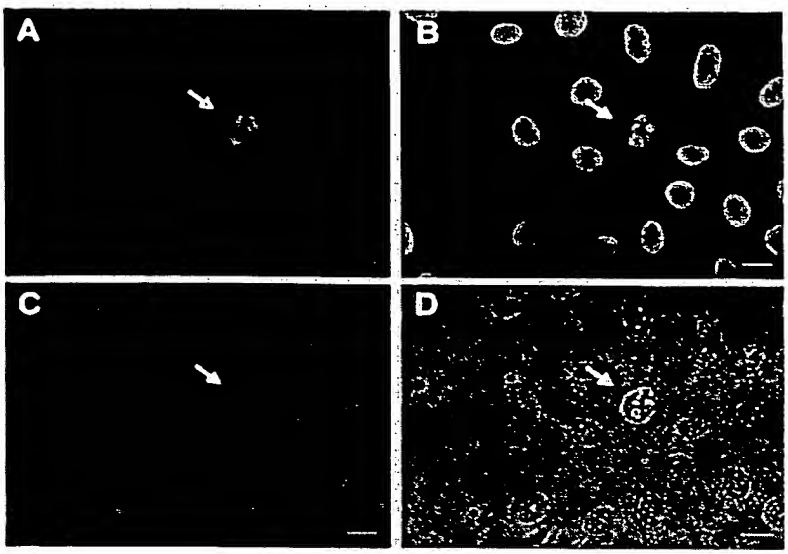

Fig. 4. Expression of kin17ΔHR protein disrupts the distribution of newly synthesized DNA. HeLa cells were transfected with pCMVKin17ΔHR. Thirty two hours later, cells were incubated in medium containing 10 μg/ml BrdU for 16 hours and double immunostained as described in Fig. 3 using anti-kin17 (pAb2064) for detection of kin17ΔHR (A) and anti-BrdU antibodies to visualize DNA replication (B). All the cells producing kin17ΔHR presented nuclear deformations but less than 20% of cells synthesized DNA at a low rate. In these cells, BrdU-staining was localized in and around large regions stained by antibodies recognizing kin17ΔHR protein (A and B). The DNA visualized using DAPI reflects a similar distribution to BrdU-staining (C). These staining patterns were associated with the morphologically abnormal intranuclear structures formed after the production of kin17ΔHR protein as judged by phase contrast analysis (D). Bars, 20 μm.

al., 1997a,b). Since the intracellular concentration of kin17 protein is low, overexpression seemed an interesting way of gaining insight into its biological role. Indeed, the levels of *Kin17* RNA in mouse adult tissues are low compared with the expression of GAPDH or β-actin RNAs. Furthermore, in cultured rodent cells, serum-stimulated proliferation, ultraviolet or ionizing-irradiation led to a 4-fold increase in kin17 protein levels (Biard et al., 1997; Kannouche et al., 1998). Therefore, the cellular concentration of kin17 protein required during proliferation or after DNA-damage remains

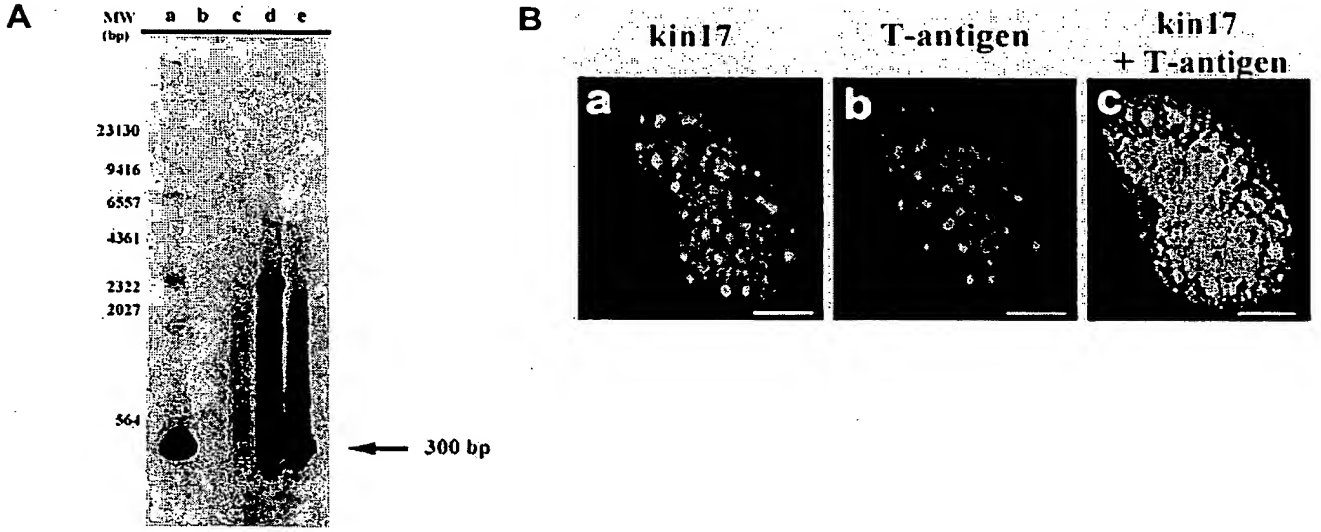

Fig. 5. Overexpression of kin17 protein in H-G1 cells reduces SV40-dependent DNA replication. (A) H-G1 cells whose genome contains the p205-GTI plasmid carrying an active SV40 origin of replication were co-transfected with 2 μg of the plasmid pKMT11 encoding the T-antigen (lane e) and with 6 μg of the plasmid pCMVKin17 (lane c) or with the plasmid pCMVDT21, missing the *Kin17* ORF (lane d). Sixty hours after transfection, low-molecular-mass DNA was extracted, digested with *Pvu*II and subjected to Southern blotting analysis with a fragment of the p205-GTI plasmid as probe. In parallel, purified p205-GTI DNA was digested with *Pvu*II and analyzed as above (lane a). Lane b was loaded with low-molecular-mass DNA extracted from mock transfected cells. The sizes of molecular mass markers are indicated as numbers of base pairs on the left in A. The arrow indicates the expected band from low-molecular-mass DNA generated by T-antigen-initiated replication of the integrated p205-GTI plasmid. (B) Detection of kin17 protein and SV40 T-antigen in H-G1 cells. Indirect immunofluorescence was carried out 22 hours after co-transfection of H-G1 cells with pKMT11 and pCMVKin17 using anti-kin17 (pAb2064, a) and anti-T-antigen (mAb 416, b) following the protocols described in Materials and Methods. The superposition of the red staining corresponding to the location of T-antigen with the green staining due to kin17 protein indicate that T-antigen is associated with kin17 foci (c). Bars, 5 μm.

relatively low. Moreover, kin17 protein is concentrated in intranuclear foci and undergoes a dynamic relocalization during the cell cycle, suggesting that kin17 protein is part of a nuclear network required during cell growth (Kannouche et al., 1997; P. Kannouche et al., unpublished results). Nevertheless, the precise intranuclear compartmentalization of kin17 protein remains to be determined since these foci do not overlap with the sites of DNA-replication, as shown by immunofluorescence detection of BrdU incorporation (P. Kannouche, unpublished results).

Here we show that the overexpression of kin17 protein results in large intranuclear structures which prevent S-phase progression, indicating that the disruption of the normal distribution pattern of kin17 foci affects DNA synthesis and is lethal for mammalian cells. We postulate that kin17 protein binds tightly to the nuclear matrix through a C-terminal domain (P. Kannouche, unpublished results), thus altering the chromatin organization. Thus, the marked modification of kin17 compartmentalization could lead to a disruption of the nuclear network, thereby possibly explaining the inhibition of cell proliferation (Kannouche et al., 1997). This hypothesis is further supported by the fact that kin17 protein colocalizes with the viral protein large T-antigen (T-Ag). Indeed, it has been shown that T-Ag is associated with the nuclear matrix in nuclear hnRNP network including peri- and interchromatin RNP fibrils which are the centers of pre-mRNA splicing (Puvion et al., 1988). Since T-Ag seems to participate in mRNA biogenesis, the colocalization with kin17 protein may indicate that both are part of the multicomponent RNA-protein complex.

The inhibition of DNA synthesis observed after overexpression of kin17 protein could be explained by the fact that the large amount of exogenous kin17 protein reduces DNA synthesis by its preferential binding to curved DNA regions. In this way it would poison the replication complex by blocking its access to DNA or slow down the propagation of replication forks. This hypothesis is supported by the following observations: (1) the transient expression of kin17 protein inhibits T-antigen-dependent DNA replication, suggesting that kin17 protein alters T-Ag activity in the early steps of initiation of DNA replication. Indeed, kin17 protein binds preferentially to curved DNA of the SV40 origin of replication and interferes with the binding of T-Ag (Deb et al., 1986; Hagerman, 1990; Mazin et al., 1994b). (2) The overexpression of H-NS protein, a functional analogue of kin17 protein which also binds to curved DNA, strongly inhibits transcription and induces the compaction of nucleoids leading to cell death (Spurio et al., 1992). (3) The overexpression of other proteins involved in DNA metabolism, as DnaC or the helicase IV of HSV, has similar lethal effects (Malik and Weller, 1996; Skarstad and Wold, 1995). However, we cannot discount the possibility that large amounts of kin17 protein lead to the formation of abnormal aggregates which will sequester essential replication proteins and indirectly perturb DNA synthesis. Taken together, these considerations indicate that kin17 protein participates in a DNA transaction that seems to be related to DNA replication.

The nuclear compartmentalization of kin17 protein appears to be essential for its biological function, as previously suggested by the analysis of different mutants of *MmKin17* cDNA (Kannouche et al., 1997). Indeed, kin17ΔHR protein produces the greatest deleterious effects compared with the wild-type kin17 protein. This indicates that *MmKin17ΔHR* cDNA could be considered as a dominant mutant in the sense that the encoded protein is hyperactive as compared with the wild-type gene product (Herskowitz, 1987). Since the absence of the central core of kin17 protein favors the formation of large structures, we hypothesize that this domain negatively regulates the binding of kin17 protein to a nuclear structure, perhaps the DNA. The central core of kin17 protein contains the RecA homologous region. In RecA protein, this motif regulates the RecA-DNA interaction. In spite of their functional role, the amino acid sequences of the C-terminal regions of bacterial RecA proteins are less conserved than the core regions (Aihara et al., 1997). Nevertheless, in different bacterial species the C-terminal DNA-binding domain is surrounded by acidic residues supposed to regulate protein-DNA interactions (Tateishi et al., 1992). Interestingly, in kin17 protein, the domain homologous to RecA is also flanked by eleven glutamic residues and two aspartic residues over 39 amino acids suggesting that it could play an analogous role. Two mechanisms may therefore account for the behavior of kin17ΔHR protein: (a) the loss of the regulatory region homologous to RecA decreases the negative charges that repulse the phosphate groups of DNA, thereby generating a kin17ΔHR protein that has enhanced DNA-binding capacities, and (b) the deletion of the core of kin17 protein abrogates the recognition of curved DNA (Mazin et al., 1994b), leading to random nonspecific binding to the chromatin. In both cases kin17ΔHR protein overcomes the endogenous kin17 activity. The deletion of the C-terminal end of kin17 protein suppresses the formation of large intranuclear structures suggesting that this domain interacts with the nuclear matrix. This idea is supported by the following properties of kin17ΔCT protein: (i) it fails to form DNM, (ii) it always allows DNA synthesis, and (iii) it produces a diffuse staining in which the foci are barely distinguishable. This C-terminal part of kin17 protein bears no significant homology to any eukaryotic protein sequence and lacks any canonical consensus motifs (Tissier et al., 1995). However, it has limited similarities with bacterial proteins such as DNA-direct RNA polymerase or recN, a putative recombination protein. Further investigation will be needed to elucidate whether these homologies have any biological significance.

The assumed participation of kin17 protein in an intranuclear network required during cell proliferation led us to test the possible relation of kin17 protein to a DNA-replication transaction. Although overexpression of kin17 protein inhibits T-Ag-dependent DNA replication, the precise molecular mechanism by which kin17 protein inhibits cell growth remains to be elucidated. The isolation of cells producing a tagged kin17 protein in which the expression could be modulated on purpose will be useful to specify further the relationship between kin17 protein and cell proliferation.

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